Genetic variation and environmental effects on beta-conglycinin and glycinin content in Brazilian soybean cultivars

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Abstract – The objective of this work was to determine genetic and environmental effects on beta-conglycinin and glycinin content in Brazilian soybean cultivars. The concentrations of these protein fractions were analyzed by scanning densitometry after electrophoresis, in 90 Brazilian soybean cultivars sown in Ponta Grossa, PR, in 2001. The effects of the sowing location were determined in the cultivar MG/BR 46 (Conquista), sown in 16 locations of Goiás and Minas Gerais states (Central Brazil), and in the cultivar IAS 5, sown in 12 locations of Paraná and São Paulo states (Southern Brazil), in 2002 soybean season. A significant variability for beta-conglycinin (7S) and glycinin (11S) protein fractions ratio was observed among the 90 Brazilian soybean cultivars. 'MS/BRS 169' (Bacuri) and 'BR-8' (Pelotas) presented the highest and the lowest 11S/7S ratios (2.76 and 1.17, respectively). Beta-conglycinin protein fractions presented more variability than glycinin protein fractions. Grouping test classified 7S proteins in seven groups, 11S proteins in four groups, and protein fraction ratios (11S/7S) in nine groups. Significant effect of sowing locations was also observed on protein fractions contents. There is a good possibility of breeding for individual protein fractions, and their subunits, without affecting protein content.

Index terms: eletrophoresis, nutraceutic properties, protein fractions, seed, sowing location.

Variação genética e ambiental e teores de beta-conglicinina e glicinina em cultivares de soja brasileiras

Resumo – O objetivo deste trabalho foi avaliar os efeitos da variação genética e ambiental sobre os teores de beta-conglicinina e glicinina em cultivares de soja brasileiras. A concentração dessas frações protéicas foi determinada por densitometria após eletroforese, em 90 cultivares de soja, semeadas em Ponta Grossa, PR, em 2001. Os efeitos dos locais de semeadura foram determinados na cultivar MG/BR 46 (Conquista), semeada em 16 locais de Goiás e Minas Gerais, e na cultivar IAS 5, semeada em 12 locais no Paraná e em São Paulo, em 2002. Foi observada variabilidade significativa quanto à razão entre as frações protéicas da beta-conglicinina (7S) e da glicinina (11S), entre as 90 cultivares avaliadas, em que a 'MG/BR 169' (Bacuri) apresentou a maior razão 11S/7S (2,76) e a 'BR-8' (Pelotas) a menor (1,17). As frações protéicas de beta-conglicinina apresentaram maior varibilidade do que as de glicinina. A análise de agrupamento discriminou as proteínas 7S em sete grupos, as 11S em quatro, e as razões 11S/7S em nove. Efeito significativo dos locais de semeadura também foi observado sobre os teores das frações protéicas. Existe uma boa possibilidade de melhoramento para as frações individuais de proteínas e suas subunidades, sem que o teor de proteína seja alterado.

Termos para indexação: eletroforese, propriedades nutracêuticas, frações protéicas, semente, local de semeadura.

Introduction

The major storage proteins of soybean seeds are beta-conglycinin (7S globulin) and glycinin (11S globulin). Beta-conglycinin is a trimeric protein composed of three subunits alpha', alpha and beta (Thanh & Shibasaki, 1977). Glycinin is a hexameric protein composed of five subunits A1aB1b, A1bB2, A1B1a, A3B4 and A5A4B3, and each subunit is composed of acidic and basic polypeptides, which are linked together by a disulfide bond (Kitamura et al., 1976; Staswick et al., 1984). Protein fractions have different functional properties, related to gel formation, thermal stability and emulsification (Utsumi et al.,

1997). Heat and calcium-induced coagulum (tofu gel) made from high content glycinin (11S) soybean is harder than with higher 7S fraction soybean (Saio et al., 1969). The large number of disulfide bonds (–SH), due to more methionine and cystein in glycinin fraction, improves gel formation capacity (Yamauchi et al., 1981) making glycinin gel harder and more turbid than beta-conglycinin gel. However, the higher hydrophobicity, the easy unfolded structure, and less thermally stable properties of beta-conglycinin make its emulsifying ability stronger than that of glycinin (Fukushima, 2001).

Genetic studies revealed variation on protein fractions and subunits, and cultivars with high levels of 7S or 11S fractions are available (Tsukada et al., 1986; Takahashi et al., 2000; Ogawa et al., 1989). The cultivar Tohoku 124 presents high ratio of glycinin to beta-conglycinin (Takahashi et al., 2000), while the cultivar Kyu-kei 305 contains only glycinin as storage proteins and lacks the three major allergenic proteins of 28K, alpha and beta subunits (Takahashi et al., 2000). Kyu-kei is the cultivar with the least quantities of allergens. Protein contents of these cultivars were the same as the recurrent parents. This tendency was also observed by Harada et al. (1983), who reported no correlation between 11S/7S ratio and seed protein content, observing no significant differences in protein content among three genotypes. Yaklich (2001), however, reported that high-protein lines appear to contain more beta-conglycinin and glycinin than normal-protein soybean lines, and the amount of subunits and polypeptides was different among lines. These conclusions were observed from data of several breeding lines evaluated for five years. Moraes et al. (2006) observed, in two high protein soybean isolines, increasing of 11S proteins and no changes in 7S proteins, which consequently promoted higher 11S/7S ratio.

Environmental effects on these protein fractions have been reported (Murphy & Ressureccion, 1984; Fehr et al., 2003). In a study of interaction between genotype and environment, Fehr et al. (2003) observed significant differences among all traits (protein fractions), except for the A3 subunit of glycinin, in 14 genotypes grown in 8 locations for three years. They observed no significant interactions between genotypes and years or locations. Murphy & Ressureccion (1984) reported differences for glycinin contents within years. Helms et al. (1998) found a genotype x environment interaction for protein content and 11S/7S ratio. The magnitude of the differences between the two cultivars for 11S/7S ratio varied among locations within year.

Soybean proteins have also been claimed to be effective in the prevention of cardiovascular diseases (USFDA, 1999), and beta-conglycinin (7S) has a role in the upregulation of liver high-affinity LDL receptors (Lovati et al., 1998). Duranti et al. (2004), investigating the effect of daily administration of isolated soybean 7S globulin alpha', observed a significant reduction on plasma cholesterol and triglycerides levels in hypercholesterolemic rats. The same authors showed that it is possible to enhance the lipid lowering effects by further increases in the alpha' subunit doses. Estimated amounts of alpha' subunit in the 7S globulin trimer is about one third of the total globulin weight (Duranti et al., 2004).

In Brazil, soybean is grown in a wide range of environments, and differences for protein fractions among Brazilian cultivars have not been analyzed. Information about differences in the amount of protein fractions among soybean cultivars could aim at processors interested in specific products, which need emulsifying or gel formation properties. Those information could also be interesting to process functional foods, since there is a promise of biological effects on lipid-lowering therapy.

The objective of this work was to determine genetic and environmental effects on beta-conglycinin – 7S (alpha', alpha, and beta subunits) and glycinin – 11S (acidic and basic subunits) contents in Brazilian soybean cultivars.

Materials and Methods

In a first experiment, 90 Brazilian soybean cultivars were analyzed to determine genetic differences on contents of protein fractions. They were sown in plots of 4 rows (5 m length), in fertile soil, in Ponta Grossa, Paraná state (25º5'S), in 2001 crop season.

In a second experiment, effects of the sowing location were determined in the cultivar MG/BR 46 (Conquista), sown in 16 locations (Anápolis, Cerrados, Senador Canedo, Alvorada, Cristalina-1, São Miguel do Passa Quatro, Rio Verde, Uberaba, Conquista, Uberlândia, Luziana, Sacramento, Buritis, Iraí de Minas, Cristalina-2 and Chapadão do Céu) of Goiás

and Minas Gerais states (Central Brazil), in 2002 crop season. Cristalina-1 and Cristalina-2 locations are different fields of the same location, and it was observed a high infestation of nematodes in the field Cristalina-2. Effects of the sowing location was also determined in cultivar IAS 5, sown in 12 locations (Guaíra, Pedrinhas Paulista, Pirassununga, Londrina, Morro Agudo, Cambará, Ibirarema, Ponta Grossa, Mandaguaçu, Cascavel, Luiziana and Nuporanga) of Paraná and São Paulo states (Southern Brazil), in 2002 crop season. For chemical analysis, grains of the three replications of the experiment carried out in field were mixed in same proportion to form a compost sample. The analysis in laboratory was conducted according to a complete randomized design with two replications (compost sample divided in two).

Ten grams of soybean seeds were ground in a centrifugal grinding mill, equipped with 24-tooth rotor and 0.5 mm stainless steel ring sieve, with the motor speed set at 15,000 rpm. This setting produced soybean flour with a uniform particle size of less than 0.25 mm. Soluble protein was extracted for 1 hour at room temperature, while stirring a gram of full fat soybean flour in a 1:15 (w/v) ratio with 0.2 M Tris-HCl buffer, pH 8, that contained 0.1 M beta-mercaptoethanol. The mixture was centrifuged at 10,000 *g* for 10 min at 4ºC. After the fat layer was removed, an aliquot of 1 mL of the protein crude extract or supernatant was taken from each sample. Total protein concentration of each sample was determined by the method of Bradford (1976). Storage proteins and their polypeptides in the crude extract were dissociated by adding an equal volume of 5% SDS solution and 0.1 M beta-mercaptoethanol to each sample; then, samples were placed in boiling water bath for 10 min for complete dissociation. Glycerol and bromophenol blue were added to each sample to the final concentration of 10 and 0.025%, respectively.

Proteins and their polypeptides were separated in a vertical slab gel apparatus, according to Chua (1980), with modifications. Each sample, containing approximately 80–100 µg proteins or 10 µL of protein sample, was loaded onto the gel. Proteins and polypeptides were separated using a linear gradient of 10 to 20% polyacrylamide gel. The dimension of the separating gels was 14x16x0.15 cm, with 15 sample wells in stacking gel. Blank sample wells were left between loaded samples to prevent protein cross-contamination during electrophoresis and to facilitate accurate quantification by scanning densitometry after electrophoresis. Since more than one gel was required for these protein samples, and it was necessary to obtain the same polyacrylamide composition throughout the entire experiment, identical gradient gels were cast from the same polyacrylamide solution and from the same gradient-production condition. Electrophoresis of each protein sample was carried out in duplicate, at a constant current of 10 mA per gel at room temperature, until bromophenol blue or tracking dye reached the bottom of the gel.

Gels were stained in freshly prepared dye containing 0.25% coomassie brilliant blue (w/v), 40% (v/v) methanol and 10% (v/v) acetic acid, and were destained in 40% (v/v) methanol and 10% (v/v) acetic acid. Destained gels were soaked in deionized water for at least 5 min. Each gel was compressed and dried in a dryer. Dried gels were scanned as previously described by Kwanyuen et al. (1997), with a molecular dynamics personal densitometer equipped with a HeNe laser light source. ImageQuant software for volume integration was used in data analysis, to determine total absorbance of entire protein bands. Apparent absorbance of each protein was obtained by subtracting the background absorbance from the total absorbance of the protein bands within the same gel volume. The relative amount of each protein or polypeptide was expressed as a percentage of total protein in the same gel lane. All data were reported as means of two replications.

A completely randomized design was used to evaluate protein fraction composition of soybean, in both experiments. In the experiment 1, ANOVA and Scott & Knott (1974) cluster analysis, at 5% of probability, was used for grouping means of the 90 cultivars. Principal component analysis (PCA) (Johnson & Wichern, 2007) was applied in order to have a more synthetic and informative description of the data set. The retained components consist of weighted sum of original variables in the following mathematical model: $PC_{il} = A_{il}X_l + ... + A_{in}X_n$ i = 1, 2,..., n, with nine variables (alpha', alpha, beta, total 7S, acidic, basic, total 11S, 11S/7S ratio and total fractions = 11S+7S). The coefficients were chosen in such a way that the first component PC_{il} had the largest variance. The output of PCA is a small number of uncorrelated components accounting for a substantial proportion of the sample data variance (Tchienkoua & Zech, 2004). A correlation

matrix among the variables of the protein fractions was also calculated. In the experiment 2, ANOVA and Tukey test, at 5% of probability, were used for comparing data from different sowing locations of the cultivars MG/BR 46 and IAS 5.

Results and Discussion

A significant variability for the 11S/7S ratio was observed among the 90 Brazilian soybean cultivars, and the values ranged from 2.76 ('MS/BRS 169' – Bacuri) to 1.17 ('BR-8' – Pelotas) (Table 1). The observed differences for protein fractions among cultivars make possible genetic manipulation to improve protein quality of soybean cultivars. Since glycinin (11S fraction) contains higher content of sulfur amino acids than beta-conglycinin (7S), it is possible to improve the amino acid balance of soybean protein. Therefore, different concentrations of protein fractions may enhance the nutritional value of soybean, as well as its properties for specific protein functionalities.

Table 1. Mean values of protein components (%), in seeds of 90 soybean Brazilian cultivars, and group classification (GC) by Scott-Knott test[1].

Cultivar	11S/7S Ratio	GC	Beta-conglycinin (7S) (%)								Glycinin (11S) (%)					
			Total (7S)	GC	alpha'	GC	alpha	GC	beta	GC	Acidic	GC	Basic	GC	Total (11S)	GC
MS/BRS 169 (Bacuri)	2.76	1	14.09	7	4.15	3	6.61	7	3.34	8	22.07	1	16.82	2	38.89	1
FT Cometa	2.62	2	14.02	7	3.47	4	6.12	8	4.42	7	18.63	3	18.08	1	36.71	2
IAC 100	2.55	2	14.10	7	3.95	3	6.29	8	3.86	8	19.64	2	16.31	2	35.95	2
BR 38	2.44	3	13.56	7	3.46	4	5.54	9	4.57	7	16.93	5	16.02	2	32.95	3
IAS 5	2.37	3	13.11	7	2.74	4	5.41	9	4.95	6	16.24	5	14.82	3	31.06	3
MG/BR-56 (Confiança)	2.35	3	13.39	7	3.75	4	5.45	9	4.20	7	16.48	5	14.92	3	31.41	3
IAC/PL 1	2.34	3	16.96	5	5.03	2	7.35	6	4.58	7	21.56	1	18.15	1	39.71	1
Embrapa 1 (IAS 5RC)	2.32	3	14.10	7	3.14	4	5.71	9	5.25	6	16.22	5	16.43	2	32.65	3
Viçoja	2.31	3	17.61	5	4.57	2	7.93	5	5.11	6	21.79	1	18.96	1	40.75	1
CEP 26 Umbu	2.31	3	14.55	6	3.38	4	6.19	8	4.98	6	17.20	5	16.35	2	33.55	3
Nova IAC 7	2.30	3	15.49	6	4.79	2	7.36	6	3.34	8	17.95	4	17.65	1	35.61	2
IAC 20	2.24	4	14.17	7	3.82	3	5.16	9	5.19	6	16.40	5	15.28	3	31.68	3
Dourados	2.21	4	18.36	5	5.30	1	8.93	3	4.13	7	21.55	1	19.01	1	40.56	1
Mineira	2.18	4	17.42	5	5.12	1	7.41	6	4.89	7	20.68	2	17.23	1	37.91	1
J 200	2.17	4	17.35	5	4.82	2	8.03	4	4.50	7	20.61	2	17.08	1	37.69	1
DM Rainha	2.17	4	17.22	5	4.49	2	7.71	5	5.02	6	18.69	3	18.62	1	37.32	1
FT Líder	2.16	4	13.63	7	3.63	4	5.43	9	4.57	7	14.85	7	14.50	3	29.34	4
EMGOPA 310	2.12	4	16.88	5	4.82	2	7.00	6	5.06	6	18.11	4	17.70	1	35.81	2
UFV 15 (Uberlândia)	2.12	4	16.53	5	4.55	2	7.22	6	4.77	7	18.36	4	16.70	2	35.06	2
Embrapa 19	2.11	4	15.64	6	3.56	4	6.18	8	5.91	5	17.66	4	15.31	3	32.97	3
MG/BR 54 (Renascença)	2.10	4	17.04	5	4.76	2	7.56	5	4.72	7	19.20	3	16.68	2	35.87	2
Embrapa 25	2.08	5	19.18	4	4.79	2	8.57	4	5.83	5	21.57	1	18.40	1	39.97	1
FT 19 (Macachá)	2.04	5	17.91	5	4.94	2	7.07	6	5.90	5	19.39	3	17.17	1	36.56	2
MG/BR 46 (Conquista)	2.04	5	17.48	5	5.44	1	7.01	6	5.03	6	18.96	3	16.68	2	35.64	2
Santa Maria	2.03	5	17.46	5	4.98	2	6.59	7	5.89	5	18.20	4	17.19	1	35.39	2
IAS 4	2.02	5	14.90	6	3.37	4	5.79	9	5.74	5	16.34	5	13.80	4	30.13	4
BRS 155	2.01	5	16.61	5	5.33	1	6.98	6	4.31	7	17.83	4	15.55	2	33.38	3
São Luiz	1.97	6	18.59	4	4.51	2	7.66	5	6.42	4	19.27	3	17.28	1	36.55	2
IAC 2	1.96	6	17.92	5	4.75	2	7.05	6	6.12	5	19.00	3	16.23	2	35.24	2
FT 2	1.96	6	20.50	3	4.53	2	9.40	3	6.57	4	21.06	1	19.16	1	40.22	1
MS/BRS 168 (Piapara)	1.95	6	19.45	4	5.34	1	7.37	6	6.74	4	20.13	2	17.74	1	37.87	1
OCEPAR 9 SS1	1.94	6	19.02	4	4.71	2	8.29	4	6.01	5	19.04	3	17.89	1	36.93	2
FT 9 (Inaê)	1.91	6	16.16	5	3.37	4	5.92	8	6.88	4	17.13	5	13.79	4	30.92	3
OCEPAR 14	1.91	6	17.38	5	3.55	4	7.07	6	6.75	4	18.04	4	15.11	3	33.16	3
BRS 158 (Milena)	1.90	6	16.58	5	4.19	3	7.26	6	5.14	6	16.96	5	14.58	3	31.54	3
IPB-T	1.90	6	17.30	5	4.56	2	7.21	6	5.53	5	17.53	5	15.35	3	32.88	3
OCEPAR 19 (Cotia)	1.88	6	16.52	5	3.58	4	5.86	9	7.09	3	15.39	6	15.71	2	31.10	3
União	1.88	6	16.83	5	4.17	3	6.91	7	5.75	5	16.41	5	15.25	3	31.65	3
SPS 1	1.87	6	20.58	3	4.34	3	8.65	4	7.59	2	19.95	2	18.45	1	38.40	1
IAS 3 (Delta)	1.86	6	18.73	4	4.31	3	8.13	4	6.30	4	18.78	3	15.97	2	34.75	2
OCEPAR 2 (IAPÓ)	1.85	6	15.26	6	3.12	4	6.31	8	5.83	5	15.43	6	12.80	4	28.23	4
MG/BR 22 (Garimpo)	1.85	7	18.60	4	5.41	1	8.92	3	4.27	7	18.40	4	15.94	2	34.34	2
BRS 133	1.84	7	20.68	3	5.21	1	8.81	3	6.66	4	20.42	2	17.74	1	38.16	1
BRS 132	1.84	7	13.92	7	3.52	4	6.21	8	4.20	7	13.67	7	11.91	4	25.58	4

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In soybean breeding programs, whose main objective is to obtain high quality protein, the 11S/7S ratio has been a criterion of indirect selection for this trait. Through the analysis of 11S/7S ratio data, it is possible to select high or low 11S or 7S lines or cultivars. Cultivar MS/BRS 169 (Bacuri) presented 11S/7S ratio of 2.76, which is relevant, when compared with observations of Kitamura & Kaizuma (1981), who reported 11S/7S ratio of 2.59 for the genotype Mo-shi-dou, which is the germplasm source for high levels of the 11S protein fractions. The same authors observed a ratio of 11S/7S of 1.12 for normal strains. Therefore, 'MS/BRS 169'(Bacuri) could be a genetic source to increase glycinin content for Brazilian soybean breeding programs.

'MS-BRS 169' (Bacuri) and 'BR-8' (Pelotas) presented the highest and the lowest 11S/7S ratios (2.76 and 1.17, respectively), among all the 90 genotypes. Content of beta-conglycinin (7S) protein subfractions alpha', alpha, and beta were, respectively, 4.1, 6.6, and

Table 1. Continuation

Cultivar	11S/7S Ratio	GC	Beta-conglycinin (7S) (%)								Glycinin (11S) (%)					
			Total (7S)	GC	alpha'	GC	alpha	GC	beta	GC	Acidic	GC	Basic	GC	Total (11S)	GC
Santa Rosa	1.84	7	20.37	3	5.53	1	7.84	5	7.01	3	20.02	2	17.42	1	37.44	1
DM Soberana	1.83	7	20.46	3	5.02	2	8.30	4	7.14	3	20.50	2	17.06	1	37.56	1
Embrapa 26	1.83	7	19.16	4	4.39	2	7.83	5	6.94	3	17.87	4	17.20	1	35.07	2
BR 6 (Nova Bragg)	1.81	7	20.77	3	4.97	2	8.05	4	7.75	2	20.18	2	17.47	1	37.66	1
BR 14 (Modelo)	1.81	7	18.78	4	5.12	1	7.26	6	6.40	4	18.92	3	15.07	3	33.99	2
BRS 138	1.80	7	18.15	5	4.35	3	8.12	4	5.68	5	16.46	5	16.28	2	32.74	3
RS 5 (Esmeralda)	1.79	7	20.00	3	5.85	1	8.18	4	5.96	5	18.96	3	16.92	2	35.88	2
CEP 10	1.79	7	15.93	5	3.44	4	6.53	7	5.95	5	15.54	6	13.03	4	28.56	4
Embrapa 48	1.79	7	15.23	6	3.64	4	5.84	9	5.75	5	13.86	7	13.41	4	27.26	4
IAC Holambra Stwart 1	1.78	7	21.22	3	5.00	2	7.98	5	8.25	2	20.48	2	17.37	1	37.85	1
EMGOPA 311	1.78	7	19.15	4	5.14	1	8.16	4	5.85	5	18.30	4	15.83	2	34.14	2
BRS 153	1.78	7	17.96	5	3.97	3	7.74	5	6.25	4	15.74	6	16.14	2	31.89	3
Davis	1.78	7	16.43	5	3.30	4	6.34	8	6.80	4	15.93	5	13.26	4	29.19	4
OCEPAR 15 (Paracatu)	1.78	7	20.79	3	5.37	1	8.88	3	6.53	4	19.29	3	17.63	1	36.92	2
BR 4	1.77	7	16.54	5	3.32	4	6.07	8	7.15	3	15.64	6	13.60	4	29.24	4
CD 202	1.76	7	15.24	6	3.50	4	6.13	8	5.61	5	14.90	7	11.97	4	26.87	4
BRS 135	1.76	7	20.75	3	4.98	2	7.89	5	7.88	2	19.37	3	17.22	1	36.59	2
Campos Gerais	1.73	7	17.46	5	4.12	3	6.42	8	6.92	3	15.43	6	14.69	3	30.12	4
MG/BR 58 (Segurança)	1.71	7	20.62	3	4.60	2	7.93	5	8.09	2	18.31	4	16.96	2	35.27	2
Bienville	1.71	7	21.25	3	5.02	2	8.84	3	7.39	3	19.68	2	16.61	2	36.29	2
FT 6 (Veneza)	1.69	8	17.94	5	3.32	4	6.63	7	7.98	2	17.54	5	12.72	4	30.27	4
MS/BR20 (Ipê)	1.68	8	21.46	3	5.14	1	9.06	3	7.26	3	19.82	2	16.25	2	36.07	2
MG/BR 66 (Liderança)	1.68	8	20.85	3	5.26	1	7.55	5	8.04	2	18.70	3	16.35	2	35.05	2
MS/BRS 170 (Taquari)	1.65	8	20.79	3	4.29	3	8.28	4	8.21	2	18.22	4	16.09	2	34.31	2
BRS 134	1.65	8	19.77	4	4.21	3	8.06	4	7.50	3	18.04	4	14.52	3	32.57	3
IAC 1	1.64	8	21.32	3	4.93	2	9.86	2	6.53	4	19.18	3	15.91	2	35.09	2
Ivaí	1.64	8	20.71	3	5.02	2	8.38	4	7.32	3	18.82	3	15.07	3	33.89	2
Ivorá	1.63	8	17.83	5	3.69	4	6.89	7	7.25	3	15.37	6	13.73	4	29.10	4
OCEPAR 6	1.62	8	18.63	4	4.27	3	7.74	5	6.62	4	16.98	5	13.19	4	30.17	4
IAC 16	1.62	8	18.20	5	3.80	3	6.63	7	7.78	2	14.93	7	14.54	3	29.47	4
BRS 156	1.62	8	17.90	5	4.01	3	7.32	6	6.58	4	15.77	6	13.17	4	28.94	4
KI-S 702	1.60	8	21.03	3	4.30	3	9.31	3	7.42	3	17.80	4	15.59	2	33.39	3
Cobb	1.59	8	21.25	3	4.11	3	7.63	5	9.51	1	17.90	4	15.93	2	33.83	2
Vila Rica	1.59	8	23.00	2	6.07	1	9.38	3	7.55	2	20.06	2	16.34	2	36.40	2
Embrapa 4 (BR 4 RC)	1.58	8	20.60	3	4.55	2	8.31	4	7.75	2	17.23	5	15.41	3	32.64	3
FT Jatobá	1.54	8	23.20	2	5.11	1	10.38	1	7.71	2	19.83	2	15.90	2	35.73	2
BRS 137	1.53	8	18.63	4	4.57	2	6.77	7	7.29	3	14.74	7	13.77	4	28.51	4
BR 23	1.52	8	21.76	2	4.71	2	9.16	3	7.88	2	18.12	4	14.95	3	33.06	3
BRS 62	1.50	9	25.42	1	5.52	1	10.73	1	9.18	1	20.42	2	17.78	1	38.20	1
OCEPAR 16	1.47	9	20.23	3	4.79	2	8.87	3	6.58	4	15.76	6	14.02	4	29.78	4
FT 14 (Piracema)	1.46	9	20.17	3	4.14	3	9.02	3	7.00	3	16.14	5	13.36	4	29.49	4
BR 36	1.46	9	23.39	2	4.98	2	9.28	3	9.12	1	18.45	4	15.71	2	34.15	2
FT 4	1.44	9	20.55	3	4.68	2	8.41	4	7.47	3	16.35	5	13.26	4	29.61	4
BR 12	1.44	9	21.36	3	4.76	2	9.20	3	7.40	3	16.08	5	14.62	3	30.70	3
BR 16	1.36	9	22.17	2	4.52	2	9.22	3	8.43	2	15.93	5	14.26	3	30.19	4
BR 8 (Pelotas)	1.18	9	23.35	2	5.52	1	10.15	2	7.69	2	14.52	7	12.92	4	27.43	4

(1)Groups determined by Scott-Knott cluster analysis, at 5% of probability.

3.3% for the cultivar MS/BRS 169 (Bacuri), and 5.5, 10.1, and 7.7% for 'BR-8' (Pelotas). Glycinin (11S) acidic and basic subfractions were, respectively, 22.1 and 16.8% for cultivar MS/BRS 169, and 14.5 and 12.9% for cultivar BR-8 (Pelotas) (Table 1). Fehr et al. (2003) found 11S/7S ratio of 2.04 for cultivar Vinton 81 and 1.51 for cultivar IA2021, in a study of three years in eight locations. 'Vinton 81' has good performance for tofu processing, which may be due to its high content of glycinin (11S). Because of its high 11S/7S ratio, the cultivar MS/BRS 169 (Bacuri) may have the same properties.

Strong positive correlations were observed among total protein frations [Glycinin (11S) and beta-conglycinin (7S)] with total 11S, and total 7S (Table 2). For 11S/7S ratio, inverse significant correlations were observed for beta-conglycinin (7S) subunits (alpha and beta) and for total 7S fraction, as expected. A positive significant correlation, although weak, was observed for total 11S protein fraction and 11S/7S ratio. Protein content exhibited no significant correlation with other protein components, as already observed by Harada et al. (1983) and Fehr et al. (2003). These data confirm the possibility of breeding for individual protein fractions and their subunits without affecting protein content. Yaklich et al. (2001), who studied the contribution of the two major protein fractions, in high seed protein lines of soybean, found higher concentration of these compounds in those lines than in normal-protein soybean lines.

Among the 90 cultivars sown in the same location, it was observed that beta-conglycinin (7S) protein fractions presented more variability than glycinin (11S) protein fractions. The Scott-Knott grouping test classified 7S proteins in 7 groups, 11S proteins in 4 groups, and protein fraction ratios (11S/7S) in 9 groups (Table 1). For the highest content of glycinin (11S), classified at group 1 by Scott-Knott test, it was observed a range of 40.75% (cultivar Viçoja) to 37.32% (DM Rainha). However, within the groups 1, 2 and 3, which presented the highest values for 11S/7S ratios, just cultivars MS/BRS 169 (Bacuri), IAC/PL 1 and Viçoja presented high values for glycinin content, and were in group 1 (Table 1).

Inverse relationship between total 7S protein fractions and protein ratios can be observed at the Scott-Knott grouping test, where the majority of the cultivars that presented low content of beta-conglycinin (7S) (groups 6 and 7) – which included cultivars IAC 20, FT-Líder, EMBRAPA 19, IAS 4, OCEPAR 2 (Iapó), BRS 132, EMBRAPA 48 and CD 202 – also presented low content of 7S protein fractions (Table 1), but were not in the first groups for the 11S/7S ratios. Cultivar BRS 132, in group 7, for both total 7S and 11S/7S ratio (13.92% and 1.84, respectively) because of the low content of 11S protein fraction (25.58%), was classified in group 4 for this fraction (Table 1).

Cultivars that presented the lowest 11S/7S ratios (group 9) also presented the highest values for total beta-conglycinin (7S), except for 'BRS 62', that is in group 1 for total 7S and 11S (Table 1). Cultivar BRS 62 presented the highest content of beta-conglycinin (7S) (25.4%), while cultivar IAS 5 had the lowest (13.11%); these cultivars had 11S/7S ratios of 1.50 and 2.37, respectively.

Figure 1 shows the functional relationship and closeness among the variables (protein fractions components). This two-dimensional representation

Table 2. Correlations among storage protein fractions in soybean seeds of 90 Brazilian cultivars[(1)].

Protein fractions	Alpha'	Alpha	Beta	Total 7S	Acidic	Basic	Total 11S	Ratio 11S/7S	Total 7S+11S	Protein (%)
Alpha'	1.00									
Alpha	0.71***	1.00								
Beta	0.18	0.49***	1.00							
Total 7S	0.69***	0.90***	0.78***	1.00						
Acidic	0.60***	0.44***	-0.04	0.34	1.00					
Basic	0.51***	0.30	-0.15	0.20	0.82***	1.00				
Total 11S	0.58***	0.39***	-0.10	0.29	0.96***	0.95***	1.00			
Ratio 11S/7S	-0.27	-0.58***	-0.81***	-0.75***	0.31	0.44***	0.39***	1.00		
Total 7S+11S	0.78***	0.76***	0.35***	0.74***	0.86***	0.77***	0.86***	-0.13	1.00	
Protein (%)	0.02	-0.06	-0.16	-0.10	0.21	0.08	0.16	0.21	0.05	1.00

***Significant at 0,1% of probability; N = 90.

of the whole data set displays 89.5% of the total protein fractions variance (TPFV) and provides a grouping of variables, according to their relative position in the plane defined by principal component analysis 1 (PC1) and principal component analysis 2 (PC2). The first component PC1 (horizontal axis), associated with 55.4% of the TPFV, displays strong and negative loadings on almost all the protein fractions, except for the subunit beta, which is more negative in relation to the second component PC2 (vertical axis) and the 11S/7S ratio, that is strongly positive related to second component either. Cultivars whose projections are at the end of the arrows (on crescent direction) are those that had high concentration of that protein fraction component, and, inversely, those that have extreme projections, at the decrescent direction, had less concentration of the specific protein component. Therefore, cultivar MS/BRS 169 (Bacuri) and cultivar BR-8 (Pelotas), which had the extreme values for 11S/7S ratios, are located in inverse extreme positions in the Figure 1, according to data in Table 1.

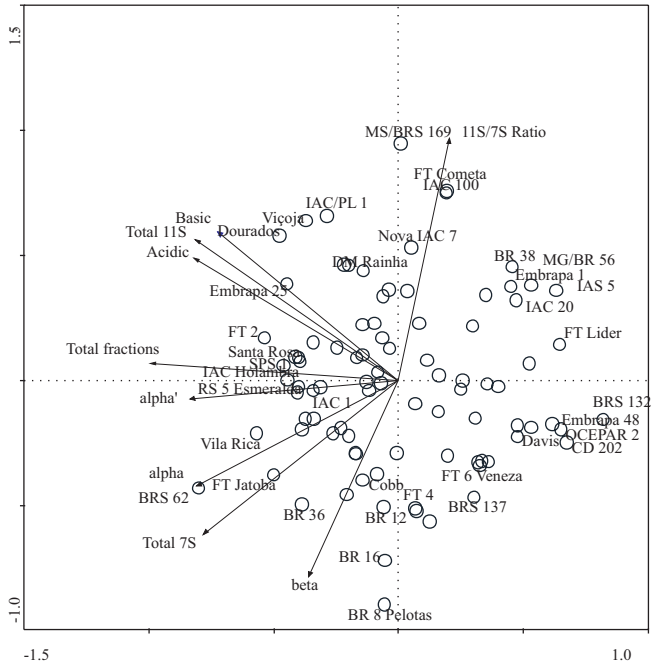

Figure 1. Principal component analysis of protein fractions and 11S/7S ratios, of 90 Brazilian cultivars, displaying the 5 highest and the 5 smallest of each fraction.

In experiment 2, when cultivar IAS 5 was sown in 12 different locations of Paraná and São Paulo states, Southern Brazil (23°S), it was observed a large variability for total glycinin (11S) and beta-conglycinin (7S) protein fractions. For total glycinin, this variability ranged from 29.6%, in Nuporanga, to 39.8%, in Pirassununga. Variability for total beta-conglycinin protein fraction ranged from 13.6%, in Londrina, to 18.6%, in Nuporanga (Table 3). Inverse relationship between 11S and 7S fractions can be observed in Nuporanga, where the highest and the lowest concentrations occurred for both protein fractions (7S and 11S) (Table 3). In this location, it was also observed the lowest value of 1.59 for the 11S/7S ratio. Cascavel was the location that presented high content of 7S and 11S protein fractions, while Londrina presented the lowest 7S concentration (Table 3). By the data of cultivar IAS 5, for 11S and 7S protein fractions concentrations and 11S/7S ratio, it can be observed that sowing location

Table 3. Glycinin and beta-conglycinin protein fraction composition (%), in seeds of soybean cultivar IAS 5, sown in 12 different locations of Paraná and São Paulo states[(1)].

Locations	Beta-Conglycinin (7S)				Glycinin (11S)			11S/7S Ratio	Total 7S+11S
	Alpha'	Alpha	Beta	Total 7S	Acidic	Basic	Total 11S		
Nuporanga	3.98a	6.64abc	7.93a	18.56a	15.34d	14.27b	29.61c	1.59d	48.17ab
Cascavel	4.93a	7.27a	5.95b	18.16ab	19.23ab	17.86ab	37.09ab	2.04bc	55.25ab
Mandaguaçu	4.73a	7.03ab	5.80b	17.58abc	18.67abc	18.36ab	37.03ab	2.10abc	54.61ab
Luiziana	4.45a	7.01ab	5.69bc	17.16abc	16.84bcd	15.91ab	32.75abc	1.90c	49.90ab
Pirassununga	4.44a	6.65abc	5.99b	17.09abc	20.39a	19.38a	39.78a	2.33a	56.86a
Ponta Grossa	4.37a	6.39abc	5.25bcd	16.02abc	17.90abcd	16.70ab	34.60abc	2.16abc	50.62ab
Guaíra	4.00a	6.21abc	4.73bcd	14.94abc	18.27abc	17.04ab	35.31abc	2.36a	50.25ab
Ibirarema	4.19a	6.00abc	4.58def	14.77abc	16.40bcd	15.67ab	32.07bc	2.17abc	46.84ab
Morro Agudo	3.54a	5.70c	5.36bcd	14.61abc	17.30bcd	15.96ab	33.25abc	2.27ab	47.86ab
Cambará	4.08a	6.02abc	4.16ef	14.27bc	17.02bcd	15.04ab	32.07bc	2.24ab	46.34ab
Pedrinhas	3.69a	5.55c	4.43def	13.69c	16.80bcd	15.34ab	32.15bc	2.35a	45.83b
Londrina	4.05a	5.92bc	3.59f	13.58c	16.29cd	15.02ab	31.31bc	2.30ab	44.89b

[(1)]Means followed by the same letters, in the columns, do not differ by Tukey test, at 5% of probability.

affected concentrations of these compounds. High differences for 11S fractions were also observed in Cascavel, Mandaguaçu, Ponta Grossa and Guaíra. In 12 locations in the Southern region of Brazil, the range for 11S/7S ratio was 2.36 to 1.59, which was similar to findings of Fehr et al. (2003), who reported differences among year-location combinations.

Data of individual beta-conglycinin subunits (Table 3) confirm the inverse relationship of 11S/7S ratio and total 7S protein fraction. Nuporanga presented the highest values for beta subunit and total beta-conglycinin (7S), while Londrina presented the smallest concentrations for the same subunits. In Pirassununga, the cultivar IAS 5 presented the highest concentration of the acidic and basic subunits of glycinin. Larger concentrations of individual subunits for both beta-conglycinin (7S) and glycinin (11S) were observed in Cascavel (Table 3), but the 11S/7S ratio was smaller (2.04), as compared to other locations. Londrina presented low concentrations of acidic and basic subunits of glycinin, and the lowest value for beta-conglycinin subunits, although the 11S/7S ratio was not the smallest (2.30). Londrina presented low precipitation (121 mm) during the filling period months (February, March and April) in 2002, while Nuporanga had good water availability (455 mm) during the same period (www.agritempo.org.br – September 2006). Observing data of Londrina and Nuporanga, it appears that beta-conglycinin protein fractions may be more susceptible to water stress than glycinin fractions.

Larger variability for beta-conglycinin than for glycinin protein fractions was observed, when cultivar MG/BRS 46 Conquista was sown in 16 locations of Central Brazil (17ºS) (Table 4). Among these locations, total 7S protein fractions ranged from 16.3%, in Rio Verde, to 24.9 % in Cristalina-1, and 11S/7S ratio was 1.89 and 1.96, respectively. Total 11S protein fractions ranged from 26.4%, in Chapadão do Céu, to 39.4%, in Senador Canedo, while 11S/7S ratio for both locations was 1.28 and 2.00, respectively. In Rio Verde, concentrations of both protein fractions, beta-conglicinin and glycinin, were reduced (Table 4). The highest values for glycinin, acidic and basic subunits were observed for sowings in Senador Canedo. The inverse relationship between total 7S and 11S/7S ratio can be observed from data of Anápolis, which exhibited lower concentrations of beta-conglycinin subunits and higher concentrations of glycinin.

Genetic variability was observed among Brazilian soybean cultivars, as well as effects of the environment on total and individual protein fractions and their subunits. However, in the present study, due to lack of specific observations on temperatures, precipitation and light incidence, it was not possible to identify which environmental component had stronger impact on concentration

Table 4. Glycinin and beta-conglycinin protein fraction composition (%), in seeds of soybean cultivar MG/BRS 46 (Conquista), sown in 16 locations of Goiás (GO) and Minas Gerais (MG) states in Central Brazil. Soybean season 2001/2002[(1)].

Locations	Beta-Conglycinin (7S)				Glycinin (11S)			11S/7S	Total
	Alpha'	Alpha	Beta	Total	Acidic	Basic	Total	Ratio	7S+11S
Cristalina 1	7.08a	8.57a	9.21a	24.86a	18.77ab	15.78bc	34.55ab	1.96bc	59.42a
Iraí de Minas	5.44bc	8.28ab	8.48ab	22.21b	17.02bc	13.76cd	30.79bc	1.38g	53.00abcd
Sacramento	6.18ab	8.37ab	6.31cd	20.87bc	18.79ab	15.74bc	34.52ab	1.65f	55.39abc
Chapadão do Céu	5.16bcd	6.47efg	9.00a	20.63bcd	13.99cd	12.41d	26.40c	1.28g	47.04d
Uberlândia	6.18ab	7.70abcd	6.42cd	20.30bcd	19.58ab	15.74bc	35.32ab	1.73def	55.63abc
Buritis	5.47bc	7.90abc	6.53c	19.90bcde	18.04abc	13.63cd	31.68bc	1.59f	51.58bcd
Senador Canedo	6.35ab	8.22ab	5.10efgh	19.68bcdef	20.52a	18.87a	39.39a	2.00abc	59.07ab
Luziânia	4.50cd	7.12cde	7.95b	19.58bcdef	17.49bc	15.56bc	33.05b	1.69ef	52.62abcd
Uberaba	6.00ab	7.50bcd	5.54ef	19.05defg	19.30ab	16.24abc	35.55ab	1.86cd	54.60abcd
Conquista	5.80abc	7.00cde	5.33efg	18.13defgh	17.78abc	15.94abc	33.72b	1.86cde	51.86abcd
Alvorada	4.93bcd	8.05ab	4.63gh	17.61efgh	17.87abc	17.01ab	34.88ab	1.98bc	52.50abcd
Cristalina 2	5.84abc	6.79def	4.52h	17.15fgh	18.62ab	15.17bcd	33.79b	1.39g	50.94cd
S.M. do Passa Quatro	4.91bcd	5.78g	5.75de	16.44gh	16.85bc	14.87bcd	31.72bc	1.92c	48.16cd
Cerrados	5.48bc	6.57efg	4.37h	16.44gh	17.95abc	16.95ab	34.90ab	2.12ab	51.34bcd
Anápolis	5.50bc	6.01fg	4.91fgh	16.42gh	18.85ab	16.58abc	35.44ab	2.16a	51.85abcd
Rio Verde	3.86d	5.81g	6.61c	16.28gh	15.84cd	14.87bcd	30.71bc	1.89cd	46.99d

[(1)]Means followed by the same letters, in the columns, do not differ by Tukey test, at 5% of probability.

of the protein components. Genotype x environment interactions should be considered in future studies.

Conclusions

1. Among Brazilian soybean cultivars, there are genetic differences for contents of 11S (glycinin) and 7S (beta-conglycinin) protein fractions, and there is a good possibility of breeding for individual protein fractions, and their subunits, without affecting protein content.

2. 'MS/BRS 169' (Bacuri) presents the better protein quality with the highest 11S/7S ratio.

3. Sowing locations affect concentration of these compounds.

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References

BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram quantities for protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v.72, p.248-254, 1976.

CHUA, N.H. Eletrophoretic analysis of chloroplast proteins. **Methods in Enzymology**, v.69, p.434-446, 1980.

DURANTI, M.; LOVATI, M.R.; DANI, V.; BARBIROLI, A.; SCARAFONI, A.; CASTIGLIONI, S.; PONZONE, C.; MORAZZONI, P. The α' subunit from soybean 7S globulin lowers plasma lipids and upregulates liver ß-VLDL receptors in rats fed a hypercholesterolemic diet. **Journal of Nutrition**, v.134, p.1334-1339, 2004.

FEHR, W.R.; HOECK, J.A.; JOHNSON, S.L.; MURPHY, P.A.; NOTT, J.D.; PADILLA, G.I.; WELKE, G.A. Genotype and environment influence on protein components of soybean. **Crop Science**, v.43, p.511-514, 2003.

FUKUSHIMA, D. Recent progress in research and technology on soybeans. **Food Science and Technology Research**, v.7, p.8-16, 2001.

HARADA, K.; TOYOKAWA, Y.; KITAMURA, K. Genetic analysis of the most acidic 11S globulin subunit and related characters in soybean seeds. **Japanese Journal of Breeding**, v.33, p.23-30, 1983.

HELMS, T.C.; CAI, T.D.; CHANG, K.C.; ENZ, J.W. **Tofu characteristics influenced by soybean crop year and location**. 1998. Available at: <http://www.ag.ndsu.nodak.edu/ndagres/fall98/ar31198.htm>. Accessed on: 3 Nov. 2006.

JOHNSON, R.A.; WICHERN, D.W. **Applied multivariate statistical analysis**. 6.ed. New Jersey: Prentice Hall, 2007. 800p.

KITAMURA, K.; KAIZUMA, N. Mutant strains with low level of subunits of 7S globulin in soybean (*Glycine max* Merr.) seed. **Japanese Journal of Breeding**, v.31, p.353-359, 1981.

KITAMURA, K.; TAKAGI, T.; SHIBASKI, K. Subunit structure of soybean 11S globulin. **Agricultural and Biological Chemistry**, v.41, p.351-356, 1976.

KWANYUEN, P.; PANTALONE, V.R.; BURTON, J.W.; WILSON, R.F. A new approach to genetic alteration of soybean protein composition and quality. **Journal of the American Oil Chemists' Society**, v.74, p.983-987, 1997.

LOVATI, M.R.; MANZONI, C.; GIANAZZA, E.; SIRTORI, C.R. Soybean protein products as regulators of liver low-density lipoprotein receptors. I. Identification of active ß-conglycinin subunits. **Journal of Agricultural and Food Chemistry**, v.46, p.2474-2480, 1998.

MILLER, I.; HAYNES, P.; GEMEINER, M.; AEBERSOLD, R.; MANZONI, C.; LOVATI, M.R.; VIGNATI, M.; EBERINI, I.; GIANAZZA, E. Proteins of rat serum. II. Influence of some biological parameters of the two-dimensional electrophoresis pattern. **Electrophoresis**, v.19, p.1493-1500, 1998.

MORAES, R.M.A. de; JOSÉ, I.C.; RAMOS, F.G.; BARROS, E.G. de; MOREIRA, M.A. Caracterização bioquímica de linhagens de soja com alto teor de proteína. **Pesquisa Agropecuária Brasileira**, v.41, p.725-729, 2006.

MURPHY, P.A.; RESSURECCION, A.P. Varietal and environmental differences in soybean glycinin and β-conglycinin content. **Journal of Agricultural and Food Chemistry**, v.32, p.911-915, 1984.

OGAWA, T.; TAYAMA, E.; KITAMURA, K.; KAIZUMA, N. Genetic improvement of seed storage proteins using three variant alleles of 7S globulin subunits in soybean (*Glycine max* L.). **Japanese Journal of Breeding**, v.39, p.137-147, 1989.

SAIO, K.; KAMIYA, M.; WATANABE, T. Food processing characteristics of soybean 11S and 7S proteins. Part I. Effect of difference of protein component among soybean varieties on formation of tofu-curd. **Agricultural and Biological Chemistry**, v.33, p.1301-1308, 1969.

SCOTT, A.J.; KNOTT, M. A cluster analysis method for grouping means in the analysis of variance. **Biometrics**, v.30, p.507-512, 1974.

STASWICK, P.E.; HERMODSON, M.A.; NIELSEN, N.C. The amino acid sequence of the A2B1a subunit of glycinin. **Journal of Biological Chemistry**, v.259, p.13424-13430, 1984.

TAKAHASHI, M.; HAJIKA, M.; MATSUNAGA, R.; KOMATSU, K.; OBATA, A.; KANAGAE, R. Breeding soybean variety lacking β-conglycinin by introduction of *Scg* gene from wild soybean. In: INTERNATIONAL SOYBEAN PROCESSING AND UTILIZATION CONFERENCE, 3., 2000, Tsukuba. **Proceedings**. Tsukuba: The Japanese Society for Food Science and Technology, 2000. p.45-46

TCHIENKOUA, M.; ZECH, W. Statistical analysis of soil variability in a humid forest landscape of Central Cameroon. **International Journal of Applied Earth Observation and Geoinformation**, v.5, p.69-79, 2004.

THANH, V.H.; SHIBASAKI, K. Beta-conglycinin from soybean proteins. Isolation and immunological and physicochemical properties of the monomeric forms. **Biochimica et Biophysica Acta**, v.490, p.370-384, 1977.

TSUKADA, Y.; KITAMURA, K.; HARADA, K.; KAIZUMA, N. Genetic analysis of subunits of two major storage proteins (β-conglycinin and glycinin) in soybean seed. **Japanese Journal of Breeding**, v.36, p.390-400, 1986.

USFDA. Food and Drug Administration. Food labeling, health claims, soy protein and coronary heart disease; final rule. **Federer Register**, v.64, p.57700-57733, 1999.

UTSUMI, S.; MATSUMURA, Y.; MORI, T. Structure-function relationship of soy proteins. In: DAMODARAM, S.; PARAF, A. (Ed.). **Food proteins and their applications**. New York: Marcel Dekker, 1997. p.257-291.

YAKLICH, R.W. β-conglycinin and glycinin in high protein soybean seeds. **Journal of Agricultural and Food Chemistry**, v.49, p.729-735, 2001.

YAMAUCHI, F.; SATO, M.; SATO, W.; KAMATA, Y.; SHIBASAKI, K. Isolation and identification of a new type of β-conglycinin in soybean globulins. **Agricultural and Biological Chemistry**, v.45, p.2863-2868, 1981.

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